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Supplemental Information

Chronic Pharmacological mGlu5 Inhibition Corrects Fragile X in Adult Mice

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Supplemental Experimental Procedures

Animals

Wild-type and *Fmr1* knock-out mice were bred on a congenic C57BL/6J or FVB background, by mating heterozygous *Fmr1*$^{0/x}$ females and WT males. Mice on a congenic C57BL/6J genetic background were used throughout except for audiogenic seizures experiments where FVB and C57BL/6J mice were used. Animals were maintained in a 12:12 h light:dark cycle, with lights on at 6 am and experiments conducted during the light phase. Genotyping was performed as described (Dölen et al., 2007). Animal housing and experimental procedures were in line with ethical and legal guidelines, and were approved by local veterinary authorities. All experiments described were conducted with experimenters blind to genotype and drug treatment.

Drug treatment

CTEP was synthesized at Roche and formulated as a microsuspension in vehicle (0.9 % NaCl, 0.3% Tween-80). Chronic treatment consisted in once per 48 h dosing at 2 mg/kg per os (p.o.) in a volume of 10 ml/kg. In animals younger than P30, acute dosing (for LTD and AGS experiments) was s.c.

Characterization of CTEP pharmacological properties

The concentration of CTEP in the plasma or brain of treated animals was determined using a combined HPLC/MS method as described in Lindemann et al. (2011). In vivo mGlu5 receptor occupancy was evaluated with [^{3}H]-ABP688 as described in Lindemann et al. (2011). Simulation of the plasma levels and receptor occupancy during chronic dosing was performed in GastroPlus software version 6.1 (Simulations Plus, Inc., Lancaster, CA, USA) using a compartmental pharmacokinetic model linked to an Emax model for occupancy estimation. The plasma pharmacokinetics for multiple dosing was simulated with a model fit to single dose data and verified to match the sparse concentration measurements made during the chronic study. The occupancy model used parameters estimated from plasma levels and occupancies measured in the vivo binding study (Emax = 92%, EC_{50} = 12.1 ng/ml).

Metabolic labeling

Metabolic labeling was performed essentially as described in Osterweil et al. (Osterweil et al., 2010). Briefly, 500 µm thick hippocampal slices were prepared from 4-week old animals and incubated at 32.5°C in ACSF (124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$ and 10 mM dextrose, saturated with 95% O_2 and 5% CO_2). Following a 3.5 h recovery period, actynomycin D at a final concentration of 25 µM and either CTEP at a final concentration of 10 µM or vehicle were added, and the slices were incubated for 30 min. A mix of [^{35}S]-labeled amino-acids (Express protein labeling mix, Perkin-Elmer) was added to the bath at a concentration of 9.5 µM (11 µCi/ml). Slices were incubated for 30 min after which the incorporation of radioactive amino acids was stopped by transferring the slices into ice-cold ACSF. The sections were homogenized in protein lysis buffer (10 mM HEPES pH7.4, 2 mM EDTA, 2 mM EGTA, 1% TX100 and protease inhibitor (Roche, Complete), and unincorporated amino acids were removed by precipitating proteins in the homogenate with trichloroacetic acid. The incorporated radioactivity was measured by liquid scintillation counting with quench correction and normalized to protein concentration and to the specific radioactivity of the reaction medium.

Electrophysiology

Fmr1 KO and wild-type littermate controls were treated acutely (s.c., 24 hours before euthanasia) or chronically (every 48 hours p.o. for 4-5 weeks) with CTEP or vehicle. 350 µm thick transverse hippocampal slices were prepared in ice-cold dissection buffer (in mM: 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 75 sucrose, 10 dextrose, 1.3 ascorbic acid), and the CA3 region was removed. Slices were left to recover for >3 hours at 32ºC in ACSF (in mM: 124 NaCl, 5 KCl, 1.23 NaH_2PO_4, 26 $NaHCO_3$, 10 dextrose, 1 $MgCl_2$, 2 $CaCl_2$) before recordings. Extracellular field potentials were recorded in stratum radiatum of CA1 in response to Schaffer collateral stimulation. Evoked responses (initial slope) were measured every 30 seconds for a 20 minute baseline, and 50 µM DHPG ((RS)-3,5-dihydroxyphenylglycine) was applied for 5 minutes to induce LTD. Experiments where baselines drifted more than 5% over 20 minutes were excluded. Maximal transient depression (MTD) for a slice was defined as

the time point post-DHPG application with the greatest depression within each individual slice. P25-P30 mice were used for acute experiments, and P58-P65 mice were used for chronic experiments. For clarity of presentation, each two points (one minute) were averaged together and represented as a single point.

Inhibitory avoidance

The inhibitory avoidance learning and extinction test, a single trial aversive conditioning followed by combined test and extinction sessions, was performed in computer-controlled shuttle boxes (San Diego Instruments, USA). All sessions started with a 90 s habituation to the lit compartment before the door separating the two compartments opened. Once the animal entered the dark compartment, the door was closed and the animal received a single electric foot-shock of 0.5 mA intensity and 2 s duration. Experimental sessions consisted in measuring the latency to enter the dark compartment 6 h, 24 h and 48 h after conditioning. The 6 h and 24 h test sessions were combined with extinction sessions during which the animals were enclosed in the dark compartment for 200 s but did not receive a foot-shock.

Whole body startle response to auditory stimuli

The whole body startle response to low intensity auditory stimuli was measured using startle response boxes (SR-LAB, San Diego Instruments). After 5 min habituation to the boxes, mice were presented with white sound pulses of 20 ms duration and moderate intensity (72 (+6), 78 (+12), 84 (+18), 90 (+24) dB over a white background noise at 66 dB). 32 pulses (8 per intensity) were presented in random order with variable inter-trial intervals (10 to 20 s).

Locomotor activity

Spontaneous locomotor activity was recorded during 10 min in large plexiglas chambers (40 x 40 cm) equipped with an automated video tracking system (Ethovision, Noldus).

Audiogenic seizure

Susceptibility to audiogenic seizure was tested in *Fmr1* KO and WT animals on the C57BL/6J and FVB genetic background. C57BL/6J mice were tested between P18 and

P22, and FVB mice were tested between P30 and P60. All animals received vehicle or CTEP at 2 mg/kg (p.o. in FVB, s.c. in C57BL/6J) 4 h before testing. Following 1 min habituation to the behavioral chamber, animals were exposed to a 120 dB sound emitted by a personal alarm siren (modified personal alarm, Radioshack model 49-1010, powered from a DC converter). Seizures were scored for incidence during 2 min or until animals reached one of the AGS endpoint (status epilepticus, respiratory arrest, death).

Golgi analysis

Golgi staining was performed in collaboration with Frimorfo Ltd. (Marly, Switzerland) on unfixed whole brains essentially as described by Gibb and Kolb (1998). Analyses were performed on apical and basal dendrites of the layer 3 pyramidal neurons in the binocular region of the primary visual cortex, and spine density was counted per segments of 25 µm, beginning at the branching point of the first branch, as described in Dölen et al. (2007).

Western blot analysis

Cortices were rapidly dissected in ice-cold ACSF, snap-frozen in isobutane on dry-ice and stored at -80°C until processing. Samples were homogenized with a glass Dounce homogenizer in ice-cold modified RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (Roche, Complete and PhosSTOP). Protein concentrations were determined with the BCA assay (Pierce) and adjusted for all samples. Aliquots of proteins were subjected to SDS-PAGE (NuPAGE 4-12% (ERK) and 3-8% (mTOR), Invitrogen), transferred to a nitrocellulose membrane (iBlot, Invitrogen) and processed for incubation with primary antibodies, followed by secondary antibodies labeled with infra-red sensitive fluorescent dyes, and scanned (Odyssey imager, LI-COR). The following antibodies and dilutions were used: from Cell Signaling Technology, anti-phospho-p44/42 MAPK (ERK1,2) (Thr202/Tyr204) (1/4000), anti-p44/42 MAPK (ERK1,2) (1/4000), anti-phospho-mTOR (Ser2481)(1/500), anti-mTOR (1/2000); from Sigma-Aldrich, anti-GAPDH (1/4000), anti-beta-actin (1/4000); from Rockland, anti-mouse IgG (IRDye-800DX), anti-rabbit IgG (IRDye-700DX). Signal intensity was quantified with Odyssey imager software.

Testis weight and hormone levels

Testis weight was determined from freshly dissected, unfixed tissue samples, and hormone plasma levels were measured using immunoassays from Cayman Chemicals (Tallinn, Estonia) following the manufacturer's instructions.

Neurological assessment, motor coordination and grip strength test

The neurological assessment including rotarod and grip strength tests of mice was performed as described (Higgins et al., 2001).

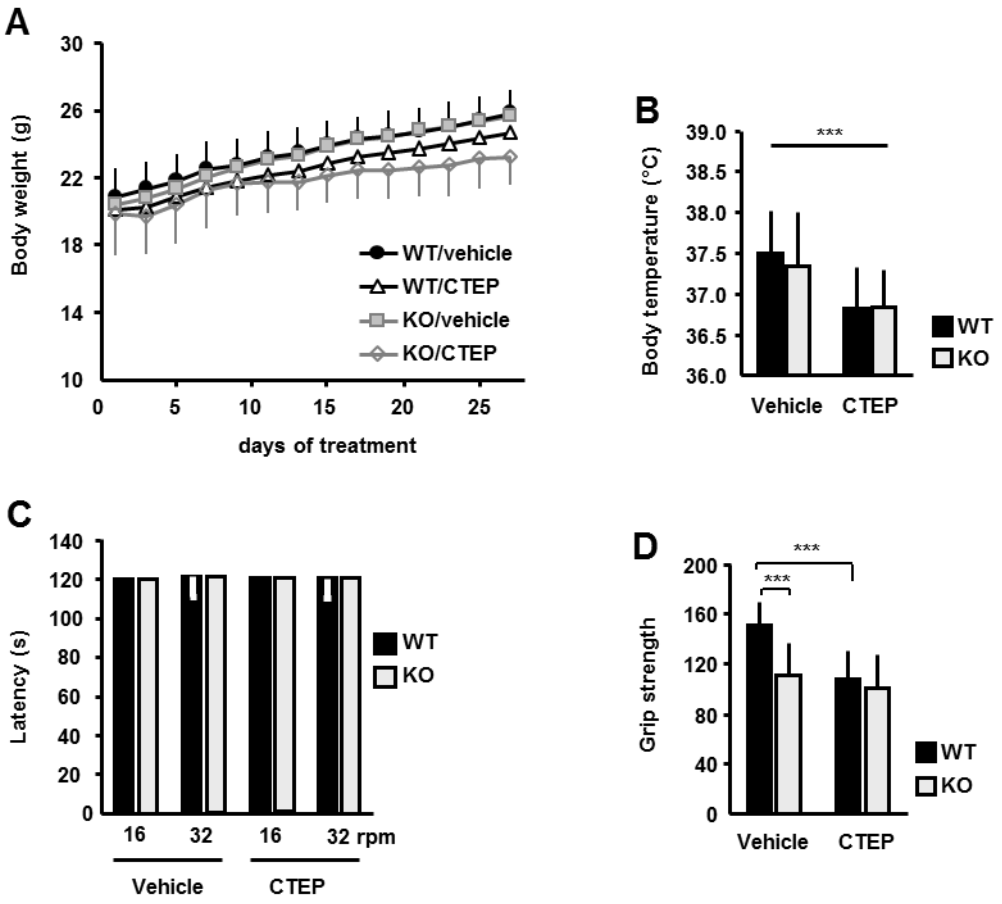

Figure S1: Effect of chronic treatment on body growth, body temperature, motor coordination, and grip strength

(**A**) **Body weight gain** during 4 weeks of chronic treatment was reduced in CTEP- compared to vehicle-treated animals, independent of the genotype. The reduction of weight gain was modest but highly significant ($p<0.0001$) when data from multiple experiments were pooled. The main effect for genotype and the interaction between genotype and treatment were not significant (genotype effect: $p=0.061$; interaction: $p=0.29$). Symbols represent mean ± SD. N=41-47 per group.

(**B**) **Body temperature** did not differ between WT and *Fmr1* KO mice treated with vehicle. Chronic treatment with CTEP for 4 weeks decreased body temperature by on average 0.5°C in both WT and KO mice (main effect for treatment $p<0.001$). Bars represent mean ± SD. N=11-12 per group.

(**C**) **Motor coordination** was evaluated on the rotarod test. There was no effect of genotype or treatment on the motor performance of vehicle- and CTEP-treated *Fmr1* KO and WT mice. Bars represent the median of each group, and error bars the lower quartile range. N=16 per group.

(D) Grip strength was significantly reduced in vehicle-treated *Fmr1* KO compared to WT littermates ($p<0.001$) at the age of 9 week. This difference was not observed after 4 weeks of chronic treatment with CTEP. The effect of treatment was significant in WT mice ($p<0.001$) but not in *Fmr1* KO mice. Bars represent mean ± SD. N=11-12 per group.

Table S1: Assessment of the general fitness of the animals

A group of 9 week old mice were tested in a battery of simple neurological and observational measures after 4 weeks of chronic treatment with CTEP. It did not reveal any alteration in the general fitness of the animals resulting from the mutation or the treatment. The scoring system is indicated in the table with the values corresponding to a normal, healthy mouse in bold. The numbers of mice are indicated between brackets.

		Scoring	WT/Veh	KO/Veh	WT/CTEP	KO/CTEP
General Observations	tremor	Yes/**No**	No (12/12)	No (11/11)	No (12/12)	No (11/11)
	eyelid closure	Yes/**No**	No (12/12)	No (11/11)	No (12/12)	No (11/11)
	piloerection	Yes/**No**	No (12/12)	No (11/11)	No (12/12)	No (11/11)
	lacrimation	Yes/**No**	No (12/12)	No (11/11)	No (12/12)	No (11/11)
	salivation	Yes/**No**	No (12/12)	No (11/11)	No (12/12)	No (11/11)
	coat dirty	Yes/**No**	No (12/12)	No (11/11)	No (12/12)	No (11/11)
Obs. In arena	transfer arousal	0-5, **3**	3 (12/12)	3 (11/11)	3 (12/12)	3 (11/11)
	body posture	0-3, **2**	2 (12/12)	2 (11/11)	2 (12/12)	2 (11/11)
	tail elevation	0-2, **2**	2 (12/12)	2 (11/11)	2 (12/12)	2 (11/11)
Reflexes	positional passivity	0-4, **0**	0 (12/12)	0 (11/11)	0 (12/12)	0 (11/11)
	righting reflex	**Yes**/No	Yes (12/12)	Yes (11/11)	Yes (12/12)	Yes (11/11)
	pinnae reflex	**Yes**/No	Yes (12/12)	Yes (11/11)	Yes (12/12)	Yes (11/11)
	cornea reflex	**Yes**/No	Yes (12/12)	Yes (11/11)	Yes (12/12)	Yes (11/11)
	flexion reflex	**Yes**/No	Yes (12/12)	Yes (11/11)	Yes (12/12)	Yes (11/11)
	startle response	0-3	1 (11/12) 0 (1/12)	1 (11/11)	1 (12/12)	1 (7/12) 0 (5/12)

Details for scoring

transfer arousal	0=coma;1=slight move;2=freezes&move;**3=normal**;4=swift;5=manic
body posture	0=flat;1=partially flat;**2=normal**;3=elevated
tail elevation	0=dragging;1=rattling;**2=normal**
positional passivity	struggles when held by: **0=tail**;1=neck;2=on back;3=hindlegs;4=none
startle response	0=none;1=flick ear;2=jump<1cm;3=jump>1cm

Table S2: Testis weight during chronic CTEP treatment

Testis weight measured before and throughout chronic treatment of mice with CTEP and vehicle, respectively. The effect sizes (lower part of the table) were calculated based on the combined analysis of the three groups of treated mice (aged 2, 3, and 6 months; N=122 in total) with a 3-way ANOVA with age, genotype and treatment as independent factors.

		Age (months)							
		1		2		3		6	
		Veh.	CTEP	Veh.	CTEP	Veh.	CTEP	Veh.	CTEP
Mean	WT	129.6	-	205.7	188.5	213.2	207	232.8	223.1
	Fmr1 KO	132.1	-	246.3	224.4	240.6	228.1	269	258.1
Stdev	WT	17	-	13.0	13.3	11.9	13.2	33.6	18.3
	Fmr1 KO	23.3	-	11.0	27.5	19.2	9.4	14.8	13.3
n	WT	11	-	11	11	10	10	9	9
	Fmr1 KO	8	-	12	11	10	11	9	9

	Effect sizes		
	mean	95% confidence interval	p value
Testis weight at 2 months of age	206.7 mg	199.8 - 213.5	
Effect of age (6 months)	+29.3 mg	21.5 - 37.1	p<0.001
Effect of the *Fmr1* KO mutation	+32.8 mg	26.5 - 39.1	p<0.001
Effect of chronic CTEP treatment	-13.5 mg	-19.8 - -7.2	p<0.001

Supplemental References

Higgins, G.A., Grottick, A.J., Ballard, T.M., Richards, J.G., Messer, J., Takeshima, H., Pauly-Evers, M., Jenck, F., Adam, G., Wichmann J. (2001) Influence of the selective ORL1 receptor agonist, Ro64-6198, on rodent neurological function. Neuropharmacology 41(1): 97-107.